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DETECTION OF YEAST MYCOFLORA FROM MILK AND YOGURT IN PAKISTAN

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Abstract

Thirty five yeast species belonging to 14 genera from milk and 16 species belonging to 9 genera from samples of yogurt were isolated, and identified on the basis of morphological and physiological/biochemical characteristics. These included teleomorphic and anamorphic ascomycetous and basidiomycetous yeast species. All yeast species appear to be new report from Pakistan. *Bullera pyricola*¸ *Candida succiphila*, *Debaryomyces castellii*, *D. hansenii* and *Pichia angusta* were predominantly isolated from samples of milk as compared to yogurt.

Introduction

Yeasts are known in the contamination and spoilage of foods and dairy products. The dairy products are favorable environment for growth of yeasts due to their acidic pH (Wickerham, 1966; Rose & Harrison, 1987). Species of *Candida*, *Debaryomyces*, *Galactomyces*, *Fellomyces*, *Pichia* and *Saccharomyces* have been isolated from different dairy products (Lodder & Kreger-Van Rij, 1952; Redhead & Malloch, 1977; Van Uden & Windisch, 1968; Wickerham, 1966; Yamada & Bannol, 1984). Yeast species mainly representatives of the genera *Candida (C. sphaerica*), *Debaryomyce*s, *Mycoderm*a, *Saccharomyces* (*S. dairensis. S. unisporu*s) and *Rhodotorula* decrease quality of dairy products by lactose assimilation (Kurtzman, 1990; Mossel, 1980; Wood, 1998). It is established that yeast species such as *C. sphaerica* ferment lactose owing to gas formation in dairy products. Their detrimental effect leads to preparing of non-quality products in the milk processing (Fleet, 1990).

There are reports of about 100 genera and 700 species of yeasts (Kurtzman & Fell, 1999) of which only 5 genera and 7 species have been recorded from Pakistan (Mirza & Qureshi, 1978). In the previous studies, yeast species were isolated and identified from soil (Mushtaq *et al*., 2004) and slime fluxes of trees (Mushtaq *et al*., 2005). The aim of this study is the isolation and taxonomic characterization of yeast species from milk and yogurt from Karachi regions of Pakistan.

Materials and Methods

Twenty samples each of milk and yogurt were collected from various localities of Karachi, Pakistan. Yeasts associated with these samples were isolated by modified serial dilution method (Harrigan & McCance, 1976). A known amount of sample was diluted up to 10,000 using double distilled sterilized water and inoculated either on malt yeast glucose peptone (YM), malt extract or yeast morphology agar medium and incubated for

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5-7 days at 25±1°C. Three isolates of yeasts per plate were selected, as representatives of the yeast mycoflora from morphologically similar looking growing colonies, which were further purified and maintained on yeast-morphology agar buffered at pH 4.5. All isolated yeasts were primarily classified into 7 different groups *viz*., pink (group I), methanol assimilating (group II), cap-, hat-, saturn- or walnut- shaped ascospore producing (group III), round-, oval-, conical- or reniform shaped ascospore producing (group IV), ballistoconidia forming (group V), basidiomycetous (group VI) and glucose fermenting (group VII). Identification of yeasts up to species level was carried on the basis of standard morphological and physiological/biochemical tests proposed for each group (Kurtzman & Fell, 1999; Barnett *et al*., 1990).

Shapes and structures of vegetative yeast cells were examined microscopically from 2-3 days old cultures growing on YM (malt-yeast-glucose-peptone) agar whereas Dalmau Plate Culture method was used to test the ability of yeast to produce pseudo- or true-hyphae and ballisto- or arthro-conidia. Thin layers of sterile corn meal agar were poured in sterilized Petri plates and dried at room temperature for 2 days before streaking with up to 4 cultures per plate. A sterile cover slip was placed over a part of each streak. After 3-5 days of incubation filamentous growth was observed in the aerobic and anaerobic (covered) portions of the streak (Beech *et al*., 1972). To observe ballistoconida formation, malt extract agar (10 to 15 ml) was poured in Petri plates and dried at room temperature for 2 days. The medium was then inoculated with the yeast to be tested in lines along two diameters at right angles. This inoculated plate was inverted over another Petri plate having a sterile microscope glass slide on the surface of the medium. The two plates were taped together all round the circumference and the preparation was incubated up to 3 weeks at 20 °C. Discharged ballistoconidia that either formed colonies on medium in the lower Petri plate or collected on the slide were examined microscopically (Barnett *et al*., 1990).

Assimilation of carbon and nitrogenous compounds were simultaneously tested in liquid yeast nitrogen base and yeast carbon base media supplemented with 50mM carbon/nitrogen source to be tested. Growth at different temperatures, in the presence of Cycloheximide (0.1% & 0.01%), and D-glucose (50% & 60%) were also tested in liquid yeast nitrogen base (used for carbon assimilation). Ability of yeast to grow without vitamin(s) was tested in liquid vitamin free yeast base. In all tests, media and reagents were prepared in double distilled sterilized water and filter-sterilized through 0.45µ filter paper using Millipore glass filtration apparatus.

Production of extra-cellular starch-like compounds was examined after a positive growth in liquid medium of a sugar or an alditol. One drop of Lugol's iodine solution was shaken with yeast culture in the tube. A blue, purple or green color indicated that the test result is positive (Cowan & Steel, 1966). Diazonium Blue B (DBB) test was tested on 10-days old culture growing on malt-yeast-glucose-peptone agar. The culture was kept at 55°C and then flooded with ice-cold DBB reagent. If the culture turned dark red within 2 min at room temperature, the result was recorded as positive (Van der Walt & Hopsu-Havu, 1976).

Results and Discussion

Thirty-five yeast species belonging to 14 genera from milk and 16 species belonging to 9 genera from yogurt were isolated and identified. Data is presented in terms of mean value of colony forming units (cfu) with standard error and range (Table 1). Identification of yeast up to species level was carried out on the basis of their morphological and physiological/biochemical characteristics (Table 2).

Table 1. Occurrence of yeast mycoflora in terms of mean colony forming units (mcfu) with standard error (se) and range, isolated from milk and yogurt.

No.	Yeast species	Milk		Yogurt	
		Occ. %	*Mcfu±se **(range)	Occ. %	*Mcfu±se **(range)
1.	*Arxula adeninovorans*	5.0	3.58±3.58^{a} (43.0)	----	---- ----
3.	*Bullera pseudoalba*	10.0	3.51±3.51^{a} (60.0-70.1)	20.0	4.46±2.84^{a} (3.1-41.5)
2.	*B. pyricola*	50.0	7.50±2.95^{b} (0.4-56.0)	----	---- ----
4.	*Candida diddensiae*	10.0	7.65±5.40^{b} (60.0-93.0)	----	---- ----
5.	*C. etchellsii*	5.0	2.00±2.00^{c} (40.0)	----	---- ----
6.	*C. haemulonii*	5.0	3.55±3.55^{a} (71.0)	----	---- ----
7.	*C. membranifaciens*	5.0	29.50±29.5^{d} (590.0)	----	---- ----
8.	*C. pseudointermedia*	5.0	3.90±3.90^{e} (78.0)	----	---- ----
9.	*C. shehatae*	5.00	1.55±1.55^{f} (31.0)	----	---- ----
10.	*C. succiphila*	40.0	9.08±5.18^{g} (3.8-78.0)	15.0	5.54±4.99^{b} (5.4-100.0)
11.	*C. valdiviana*	5.0	0.28±0.28^{h} (5.6)	20.0	0.92±0.58^{c} (0.7-8.5)
12.	*Clavispora lusitaniae*	10.0	5.75±3.97^{i} (54.0-61.0)	15.0	9.50±6.55^{d} (90.0-100)
13.	*Cryptococcus albidus*	10.0	30.50±29.46^{d} (20.0-590.0)	----	---- ----
14.	*C. gastricus*	5.00	3.00±3.00^{J} (60.0)	----	---- ----
15.	*Debaryomyces castellii*	45.0	10.11±3.64^{k} (2.5-53.0)	15.0	14.50±7.93^{e} (90.0-100.0)
16.	*D. hansenii*	50.0	13.39±5.19^{L} (2.1-71.1)	15.0	0.74±0.40^{f} (4.8-5.2)
17.	*D. vanrijii*	15.0	32.08±29.45^{m} (5.6-590.0)	----	---- ----
18.	*D. yamadae*	5.00	0.90±0.90^{n} (18.0)	----	---- ----
19.	*Fibulobasidium inconspicuum*	5.00	20.50±20.50^{o} (410.0)	----	---- ----
20.	*Lipomyces lipofer*	5.0	2.15±2.15^{c} (43.0)	----	---- ----
21.	*L. starkeyi*	10.0	3.94±3.74^{e} (3.8-75.0)	----	---- ----
22.	*Pichia angusta*	40.0	6.5±3.24^{p} (3.50-51.0)	----	---- ----
23.	*P. anomala*	25.0	2.48±1.52^{q} (2.9-30.0)	10.0	2.10±1.48^{g} (20.1-22.9)
24.	*P. heimii*	10.0	20.69±20.49^{o} (3.8-410.0)	----	---- ----
25.	*P. lynferdii*	35.0	12.87±5.85^{r} (3.8-63.8)	15.0	0.12±0.06^{h} (0.7-0.8)
26.	*P. mexicana*	----	---- ----	10.0	0.32±0.22^{i} (3.2)
27.	*P. ofunaensis*	5.0	0.38±0.38^{h} (3.0-75.0)	15.0	1.00±0.55^{c} (5.1-7.4)
28.	*P. ohmeri*	10.0	2.49±2.30^{q} (3.8-46.0)	10.0	0.32±0.22^{i} (0.7-3.1)
29.	*P. strasburgensis*	10.0	6.84±4.80^{p} (56.0-80.9)	----	---- ----
30.	*P. sydowiorum*	5.0	1.60±1.60^{f} (32.0)	----	---- ----
31.	*Saccharomycodes ludwigii*	5.0	0.19±0.19^{h} (3.8)	----	---- ----
32.	*Sporidiobolus ruineniae*	5.0	2.88±2.70^{J} (3.70-54.0)	20.0	1.06±0.53^{c} (3.2-7.4)
33.	*S. salmonicolor*	5.0	3.75±3.75^{e} (75.0)	15.0	3.5±1.92^{J} (20.0-25.0)
34.	*Sporobolomyces tsugae*	10.0	5.50±3.84^{i} (46.0-64.0)	10.0	10.0±6.88^{k} (100.0)
35.	*Stephanoascus ciferrii*	15.0	3.17±1.99^{J} (5.9-31.5)	20.0	0.10±0.05^{h} (0.3-0.7)
36.	*Tremella encephala*	5.0	1.20±1.20^{f} (24.0)	----	---- ----
37.	*Williopsis californica*	----	---- ----	15.0	1.09±0.60^{c} (7.3)

*Values are in 10,000; ** single values in parentheses indicates that yeast species was isolated only from 1 sample. Mean values in each column having different letters are significantly different at $p<0.001$ (Bonferoni test).

Among identified species, teleomorphic ascomycetous yeasts were *Clavispora lusitaniae* Rodrigues de Miranda, *Debaryomyces castellii* Capriotti, *D. hansenii* (Zopf) Lodder & Kreger-van Rij, *D. vanrijii* (van der Walt & Tscheuschner) Abadie, *et al*., *D. yamadae* (van der Walt & Johannsen) van der Walt, *et al*., *Lipomyces lipofer* Lodder & Kreger-van Rij ex Slooff, *L. starkeyi* Lodder & Kreger-van Rij, *Pichia angusta* (Teunisson, *et al*.,) Kurtzman, *P. anomala* (Hansen) Kurtzman, *P. heimii* Pignal, *P. lynferdii* (van der Walt & Johannsen) Kurtzman, *P. mexicana* Miranda, *et al*., *P. ofunaensis* (Makiguchi & Asai) Kurtzman, *P. ohmeri* (Etchells & Bell) Kreger-van Rij, *P. strasburgensis* (Ramirez & Boidin) Phaff, *P. sydowiorum* (Scott & van der Walt) Kurtzman, *Saccharomycodes ludwigii* Hansen, *Stephanoascus ciferrii* Smith, *et al*., and *Williopsis californica* (Lodder) von Arx.

Among anamorphic ascomycetous yeast species, *Arxula adeninovorans* (Middelhoven, *et al*.,) van der Walt, *et al*, *Candida diddensiae* (Phaff, *et al*.,) Fell *et al*., *C. etchellsii* (Lodder & Kreger-van Rij) Mayer & Yarrow, *C. haemulonii* (van Uden & Kolipinski) Mayer & Yarrow, *C. membranifaciens* (Lodder & Kreger-van Rij) Wickerham &. Burton, *C. pseudointermedia* Nakase, *et al*., *C. shehatae* Buckley & van Uden, *C. succiphila* Lee & Komagata and *C. valdiviana* Grinbergs & Yarrow were identified.

Teleomorphic basidiomycetous yeasts were identified as *Fibulobasidium inconspicuum* Bandoni, *Sporidiobolus ruineniae* Holzschu, *et al.*, *S. salmonicolor* Fell & Tallman and *Tremella encephala* Persoon. *Bullera pseudoalba* Nakase & M. Suzuki, *B. pyricola* Stadelmann, *Cryptococcus albidus* (Saito) Skinner, *C. gastricus* Reiersöl & di Menna and *Sporobolomyces tsugae* (Phaff & do Carmo-Sousa) Nakase & Itoh were identified as anamorphic basidiomycetes. All yeast species appeared to be new reports from Pakistan.

It may be noted that all the yeast species isolated from yogurt were also found in samples of milk except that of *Pichia mexicana* and *Williopsis californica*, however, their occurrences were lesser as compared to samples of milk (Table 1). In samples of milk, *Bullera pyricola*¸ *Candida succiphila, Debaryomyces castellii*, *D. hansenii* and *Pichia angusta* were predominant and showed 40% or more occurrences. Significant differences of occurrences were found between yeast species isolated from milk and yogurt during analysis of variances (Table 3A & B) and Bonferroni test (Table 1).

Table 3. ANOVA of yeast species isolated from dairy products.

Source	Sum of squares	df	Mean square	F	Probability
		Milk			
Main effects					
Yeasts (A)	150494.171	30	5016.472	5906.638	p<0.001
Sample (B)	154557.125	12	12879.76	15165.255	p<0.001
A*B	2615180.849	360	7264.391	8553.447	p<0.001
Error	342.265	403	0.849		
Total	3059064.25	806			
		Yogurt			
Main effects					
Yeasts (A)	14623.488	15	974.899	26605.554	p<0.001
Sample (B)	30493.84	19	1604.939	43799.697	p<0.001
A*B	13193.3	285	462.917	12633.267	p<0.001
Error	11.726	320	3.66E-02		
Total	**186158.4**	**640**			

Yeast counts (colony forming units, cfu) of most of the yeast species isolated from milk and yogurt, were between 10^3 and 10^5 cells g^{-1}. Highest yeast counts were observed in species of *Candida*, *Cryptococcus, Debaryomyces*, and *Pichia* (10^5 cells g^{-1}). Yeast counts in dairy products between 10^4 and 10^5cells g^{-1} have been reported in Australia (Fleet & Mian, 1987; Suriyarachchi & Fleet, 1981), Nigeria (Green & Ibe, 1987) and Egypt (Haridy, 1993). However examples of yeast occurrences in yogurts with more than 10^6 cells g^{-1} (Rohm *et al*., 1990; Van-Uden & Carmo-Sousa, 1957) and 10^3 cells g^{-1} or lesser have also been recorded from various countries such as UK, Canada, USA and the Netherlands (Arnott *et al*., 1974; Davis, 1975; Saad *et al*., 1987).

Several species of *Candida* and *Debaryomyces* have been reported as contaminant in milk and yogurt (Rose, 1982; Wood, 1985). They are mainly involve in deterioration (Fleet, 1990,1992) and also responsible for off-flavors and loss of texture quality due to gas production during lactose assimilation (Foschino *et al*., 1993)**.** Some representatives of the genus *Rhodotorula* cause staining and give a bitter taste to the products. For example, from the fermented cream it is hard to obtain a churned butter. From the curd, which is a secondary product of the white and yellow cheese processing, the presence of yeasts leads to the so-called "yeast taste". This reflects on the taste quality of the curd if these microorganisms exceed 10,000 per gram product (Savova & Nikolova, 2000-2002). Warmer weather, inadequate refrigeration and improper storage are the principal causes of higher levels of contamination, increased diversity and change in yeast mycoflora (Moreira *et al*., 2001).

During present studies, samples of milk and yogurt were found highly contaminated with several yeast species. Ideally, the population of yeast species in dairy products should not exceed 10 yeast cells g^{-1} and values higher than this will probably mean that the product may spoil before refrigeration (Moreira *et al*., 2001). This suggested that overall improved and high quality of hygienic precautions should be adopted to avoid contamination especially during production of yogurt.

Physiologically 4 methanol assimilating (methylotrophic) yeasts were identified as *Candida valdiviana*, *Pichia anomala*, *P. mexicana* and *P. ohmeri*. During assimilation tests, when the carbon source is switched from glucose to methanol (or alkanes) the cells of these yeast species become packed with microbodies (peroxysomes) that contain enzyme, alcohol oxidase. This characteristic makes the methylotrophic yeasts ideal vehicles for production of heterologous proteins such as harmones – somatostatin, tumor necrosis factor, and many others. It is interesting that *P. mexicana* is also reported as lipolytic yeast (Spencer & Spencer, 1997). This yeast species has very strong activity against monoesters of short-chain fatty acids (C8, C10, C12) with triterpenes and sterols.

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